

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 146 413**  
**A2**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 84308984.8

(22) Date of filing: 20.12.84

(51) Int. Cl.: **C 07 K 15/26, C 12 P 21/02,**  
**C 12 N 15/00, C 07 H 21/04,**  
**A 61 K 45/02**

(30) Priority: 21.12.83 GB 8334102

(43) Date of publication of application: 26.06.85  
Bulletin 85/26

(64) Designated Contracting States: DE FR GB IT

(71) Applicant: **G.D. Searle & Co., Box 1045, Skokie,**  
**Illinois 60076 (US)**

(72) Inventor: **Bell, Leslie David, 14 Hazel Avenue Thame,**  
**Oxon (GB)**  
Inventor: **Smith, John Craig, 2 Edgecote House**  
**Amersham Hill High Wycombe, Bucks (GB)**  
Inventor: **Porter, Alan George, 6 Disraeli Crescent High**  
**Wycombe, Bucks (GB)**  
Inventor: **Adair, John Robert, 3 Willow Way Loudwater**  
**High Wycombe, Bucks (GB)**

(74) Representative: **Goldin, Douglas Michael et al, J.A.**  
**KEMP & CO. 14, South Square Gray's Inn, London**  
**WC1R 5EU (GB)**

(54) Interferons with novel cysteine pattern.

(57) Human interferons are modified by providing novel cysteine substitutions and disulfide bonds. The amino acid sequence of a first interferon is combined with the cysteine and/or disulfide pattern of a second interferon to give modified interferons with hybrid properties. DNA polymers encoding for the modified interferons are disclosed together with plasmids incorporating the DNAs and the transformation of host cells with the plasmid. The modified interferons are expressed by the transformed host cells and can be formulated for pharmaceutical use.

**EP 0 146 413 A2**

**BEST AVAILABLE COPY**

TITLE: "INTERFERONS WITH NOVEL CYSTEINE PATTERN"

SPECIFICATION

Background of the Invention

1. Field of the Invention

This invention describes the use of recombinant DNA technology for the design of novel interferon molecules. These novel interferons comprise the amino acid sequence of human  
5 beta interferon modified by the addition or deletion of one or more cysteine residues thereby changing the disulfide cross-linking characteristics of the interferon.

2. Description of the Prior Art

INTRODUCTION

10 The novel feature of the invention described below is that the coding sequence of the human interferon beta gene (HuIFN- $\beta$ ) has been changed at specific points, by the process of site directed mutagenesis, to induce novel amino acid arrangements.

15 Specifically the number and arrangement of cysteine residues has been altered to produce a pattern analogous to that observed in the human interferon- $\alpha$  (HuIFN- $\alpha$ ) family. The novel arrangement is expected to impose an IFN- $\alpha$ -like tertiary structure on the IFN- $\beta$  protein sequence and hence  
20 lead to novel properties of the molecule.

The alpha and beta interferons, specifically Human IFN $\alpha_1$ ; (D) and Human IFN- $\beta$  have been shown to be structurally related. They are 45% homologous at the nucleotide level and 29% homologous at the amino acid level (Taniguchi et al., Nature 285 547 (1980). Sternberg and Cohen (Int. J. Biol. Macromol. 4 137 (1982)) have produced a model suggesting the  $\alpha$  and  $\beta$  interferons' tertiary structure may be similar. Disulfide bonds are known to influence both tertiary protein structure and stability. The tertiary structure of the Human IFN $\alpha$  has been shown to be in part dependent upon the disulfide linkages (Wetzel et al. UCLA Symp. Mol. Cell Biol. 1982, 25, 365-376)

The interferons are a class of proteins that occur in vertebrates and function as biological regulators of cell function which include increasing resistance to pathogens, limiting cell growth and modulating the immune system. The most studied property of the interferons is their ability to convert cells into an "antiviral" state during which they are more resistant to virus replication (Lengyel, Annual Review of Biochemistry, 51:251, 1982).

In addition to conferring antiviral resistance on target cells, interferons (IFNs) have both immunomodulatory and antiproliferative properties (Stewart, 1979, The Interferon System, Springer, Berlin). The IFNs, by virtue of their antigenic, biological and physico-chemical properties, can be

grouped into three classes: Type I, IFN- $\alpha$  ("leucocyte") and IFN- $\beta$  ("fibroblast"); and Type II, IFN- $\gamma$  ("immune") (Stewart et al., 1980, Nature, 286, 110). Detailed information is now available on the virus-induced, acid stable

5 IFN- $\alpha$  and IFN- $\beta$  and the mitogen-induced IFN- $\gamma$ . All three IFN cDNAs have been cloned from their respective induced mRNAs, the DNA sequenced and their potential protein sequences deduced (Taniguchi et al., 1979, Proc. Japan Acad. Ser. B 55, 461-469; Houghton et al., 1980, Nucleic Acids Res. 8, 2885-2894; Nagata et al., 1980, Nature, 284, 316-320; Nagata

10 et al., 1980, Nature, 287, 401-408; Goeddel et al., 1981, Nature, 290, 20-26; Gray et al., 1982, Nature, 295, 503-508). IFNs- $\alpha$  and IFN- $\beta$  have been purified to homogeneity and the partial protein sequences obtained confirm

15 the derived IFN- $\beta$  sequence and the sequences of some recombinant IFN- $\alpha$ 's (Allen and Fantes, 1980, Nature, 287, 408-411; Knight et al., 1980, Science, 207, 525-526; Stein et al., 1980, Proc. Natl. Acad. Sci, USA, 77, 5716-5719; Zoon et al., Science, 207, 527-528). The cysteine at the 17 position

20 of beta interferon has been replaced by serine (R. O'Connell, Genetic Technology News, 3: 2, July 1983, European Patent Application 83306221.9)

Human IFN- $\alpha$  is specified by a multigene family comprising at least 14 different genes, with at least 3 additional

25 pseudogenes and 4 other genes known to hybridize, but not yet sequenced (Weissman, 1982, 11th Annual UCLA Symposium on Molecular and Cellular Biology). In contrast, there is only

one well characterised human IFN- $\beta$  gene (Owerbach *et al.*,  
1981, Proc. Natl. Acad. Sci, USA, 78, 3123-3127). The IFN- $\gamma$   
gene differs from IFNs- $\alpha$  and - $\beta$  by having three introns and  
thus displays another distinction between the Type I and Type  
5 II IFNs (Gray and Goeddel, 1982, Nature, 298, 859-863).

Homologies exist between members of the human IFN- $\alpha$  multigene  
family, and between human IFN- $\alpha$  and IFN- $\beta$  genes. It  
appears that IFN- $\alpha$  and IFN- $\beta$  genes are the products of an  
ancient gene duplication, and perhaps diverged early in  
10 vertebrate evolution (Taniguchi *et al.*, 1980, Nature, 285,  
547-549). In contrast, the IFN- $\alpha$  multigene family seems to  
have diverged much more recently, perhaps within the last 26  
million years (Miyata & Hayashida, 1982, Nature, 295, 165-168).

While the mechanism of action of interferons is not completely  
15 understood, certain physiological or enzymatic activities  
respond to the presence of the interferons. These activities  
include RNA synthesis and protein synthesis. Among the enzymes  
induced by interferons is (2'-5')(A)<sub>n</sub> synthetase which is  
activated by double stranded RNA. This synthetase generates  
20 2'-5' linked oligoadenylates from ATP which activates a latent  
endoribonuclease, RNase L, which cleaves single stranded RNA  
such as messenger RNA (mRNA) and ribosomal RNA (rRNA).  
Interferon induces a protein kinase which phosphorylates at  
least one peptide chain initiation factor and inhibits protein  
25 synthesis (Lengyel, *ibid* p. 253)

Interferons have been shown to be negative growth regulators for cells by regulation of the (2'-5')A<sub>n</sub> synthetase activity (Creasey et al., Mol. and Cell Biol., 3, 780,786 1983).

5 IFN- $\beta$  was indirectly shown to be involved in the normal regulation of the cell cycle in the absence of inducers through the use of anti-IFN- $\beta$  antibodies. Similarly, interferons have been shown to have a role in differentiation (Dolei et al., J. Gen. Virol 46: 227-236, 1980) and in immunomodulation (Gresser, Cell. Immunol. 34: 406-415, 1977).

10 Interferons may also alter methylation patterns of mRNAs and alter the proportion of fatty acids in membrane phospholipids, thereby changing the rigidity of cellular membranes. These and other mechanisms may respond to interferon-like molecules in varying degrees depending upon the structure of the  
15 interferon-like polypeptide. It is envisaged that an IFN- $\beta$  with an IFN- $\alpha$  disulfide pattern may display a new advantageous phenotype. For example, IFNs which show a greater antiviral to antiproliferative activity (and vice-versa) or have an enhanced activity/specificity against a particular  
20 virus infected tissue or transformed cell mass.

Increased stability is an expected result of the creation of new disulfide bonds. Stability is defined as increased resistance to denaturation, proteolytic enzymes and other physical or activity changes. The increase in stability

results in improved recovery during production, increased storage life and prolonged activity in solution.

The design and synthesis of new interferon-like polypeptides composed of beta interferons with alpha interferon disulfide  
5 pattern may allow the selective activation of only part of normal interferon-induced activities. These hybrid polypeptides could then be used appropriately to activate the interferon systems. In addition, the affinity of these modified interferons for cell surface receptors may differ from  
10 that of naturally occurring interferons. This would allow selective or differential targeting of interferons for a particular cell type.

Novel interferons containing a modified cysteine pattern can be constructed for the cysteine pattern of alpha, beta or gamma  
15 interferons using the methods of this invention. That is, a human alpha interferon can incorporate the cysteine patterns of the human beta or human gamma interferons; a human beta interferon can incorporate the cysteine patterns of human alpha or human gamma interferons; and a human gamma interferon can  
20 incorporate the cysteine patterns of human alpha or beta interferons. Similarly, a combination cysteine pattern from two or more interferon classes can be substituted into the third interferon class.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a summary flow chart illustrating the amino acid changes by site directed mutagenesis resulting in IFNX802, 803 and 804.

5 Figure 2 illustrates the restriction map of Construction I.

Figure 3 illustrates the restriction map of Constructions II through V.

Figure 4 illustrates the site directed mutagenesis resulting in IFNX802.

10 Figure 5 illustrates the site directed mutagenesis resulting in IFNX803.

Figure 6 illustrates the site directed mutagenesis resulting in IFNX804.

Figure 7 illustrates the restriction map of Constructions VI,  
15 VII and VIII.



## DESCRIPTION OF THE MODIFIED INTERFERONS

Chart 1 illustrates the DNA sequence coding for the amino acid sequence of IFN802. In addition to the traditional three letter abbreviation for amino acids, at the bottom of the figure is the one letter abbreviations recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

Chart 2 illustrates the DNA sequence coding for the amino acid sequence of IFNX803.

Chart 3 illustrates the DNA sequence coding for the amino acid of IFNX804.

Chart 4 illustrates the DNA sequence coding for the amino acid sequence of IFNX815.

Chart 5 illustrates the DNA sequence coding for the amino acid sequence of IFNX816.

Chart 6 illustrates the DNA sequence coding for the amino acid sequence of IFNX817.

Chart 7 illustrates the DNA sequence coding for the amino acid sequence of IFNX818.

Chart 8 illustrates the DNA sequence coding for the amino acid sequence of IFNX457.

## 5 SUMMARY OF THE INVENTION

One object of this invention is the reorganization of the position and number of cysteines in HuIFN- $\beta$  to a pattern analogous to that found in the HuIFN- $\alpha$  family, by the process of site directed mutagenesis of individual nucleotides of the HuIFN- $\beta$  coding sequence, so as to cause defined changes in the amino acid sequence of the HuIFN- $\beta$ . The resultant modified HuIFN- $\beta$  molecules show different or novel properties from that of HuIFN- $\beta$ , and may show properties similar to those exhibited by the HuIFN- $\alpha$  family. A summary flow chart of the construction of the modified HuIFN- $\beta$  IFNX802, 803, and 804 molecules are shown in figure 1. Similarly, the disulfide pattern and amino acid sequence of the alpha, beta and gamma human interferons can be combined to form new hybrid or modified interferons. Another object of the invention is to create disulfide linkages that improve the physical and pharmacological properties of modified interferons, including stability.

An object of the invention is the production of DNA sequences coding for the production of the modified-interferons IFN $\alpha$  802, 803, 804, 815, 816, 817, 818 and 457 and the amino acid sequences themselves.

5 Another object of the invention is the production of a pharmaceutical composition containing a therapeutically effective amount of the modified-interferons which are useful for anti-viral, anti-proliferative, anti-tumor, immunomodulatory or immunogenic treatment. Yet another object  
10 of the invention is a method of producing the modified-interferons of the present invention utilizing microorganisms.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### I. Rationale for construction

15 The coding sequences of the leukocyte (alpha) interferon (IFN- $\alpha$ ) family are distantly related to the sequence of the fibroblast (beta) interferon (IFN- $\beta$ ) gene, for example the coding sequence of HuIFN- $\alpha_1$  (D) is 45% homologous at the nucleotide level and 29% homologous at the amino acid level to  
20 HuIFN- $\beta$  (Taniguchi et al, Nature 285 547 (1980).

Secondary and tertiary structures of  $\alpha$ - and  $\beta$ -interferons have been derived by various computer modelling

procedures. Sternberg and Cohen (Int.J.Biol.Macromol. 4 137 (1982)) have produced a model for interferon tertiary structure applicable either to alpha or beta interferon, suggesting that the in vivo structures of each type of interferon are similar.

- 5 Human interferon alpha's have been shown to contain four cysteines at positions 1, 29, 98, 138 (positions relate to the IFN- $\alpha_2$  (A) sequence) which have been shown to form two intramolecular disulfide bridges with bonds between cys 1 and cys 98, between cys 29 and cys 138 (Wetzel, Nature 289 606  
10 (1981). Human interferon beta contains three cysteines, at positions 17, 31 and 141. Positions 31 and 141 in beta have been considered analogous to positions 29 and 138 in the interferon alpha family. It has been shown that alteration of cys<sup>141</sup> to tyr<sup>141</sup> abolishes interferon beta antiviral  
15 activity. Further it has been described that pre-treatment of HuIFN- $\beta$  with the reducing agent dithiothreitol abolishes antiviral activity (Shepard et al, Nature 294 563 (1981) ). These observations have been taken to show that a disulfide bridge between cys 31 --- 141 is essential for the activity of  
20 HuIFN- $\beta$ .

The cysteine substitutions in the modified interferons may be in the analogous position of another interferon or in the same sequential amino acid position. From the same sequential position, the cysteine may be moved 1 to 4 amino acid positions  
25 toward either end of the polypeptide replacing the amino acid

normally in that position. Therefore, the cysteine substituted for the third amino acid of human beta interferon may be alternatively substituted at amino acid positions 1, 2, 4, 5, 6, or 7. Similarly, the cysteine substitution at position 101, 5 may also be at positions 97, 98, 99, 100, 102, 103, 104, or 105. These alternative position substitutions result in an altered stress level on the disulfide bond formed.

## II. Outline of the technique of site directed mutagenesis

Site directed mutagenesis, using oligonucleotides to 10 produce defined sequence alterations is perhaps the most specific means of producing mutants in vitro.

The background to the method involved is reviewed in Zoller and Smith (Nucleic Acids Res. 11(20) 6487 (1982) ), which also details many of the technical aspects of the methodology.

15 To summarise the procedure, the desired nucleotide change(s) are identified and an oligonucleotide constructed which is complementary to the region 5' and 3' to the nucleotide of interest. At this position the nucleotide used is that which will be complementary to the desired mutation 20 (e.g., Figures 4-6). Thus when the oligonucleotide is annealed to the coding sequence a mismatch occurs at the point of interest.

In order to produce the coding strand in a single stranded form the most practical means is to introduce the sequence into a bacteriophage which has both double stranded and single stranded DNA phases during its life cycle. Two phages commonly  
5 used are  $\phi$ x174 and M13. The bacteriophage M13 was used in this method.

After annealing of the oligonucleotide to the sequence to be mutated, closed circular molecules are transcribed and ligated. The complete molecules may be separated from  
10 incomplete transcripts either by separation on alkaline sucrose gradients (Zoller and Smith, 1982) or by electrophoresis and elution from low gelling temperature agarose. The latter technique has the advantage that subsequent transformation of the DNA into competent E.coli JM101 cells can be done in the  
15 presence of the agarose, as will be described, so reducing the number of manipulations.

Prior to separation of transcripts, by whichever means, incomplete transcripts may be degraded by  $S_1$  nuclease to allow greater resolution during the separation stage. After  
20 transformation a population of mutant and wild type molecules are obtained which may be distinguished by one of a number of screening techniques.

For example, the desired change may introduce or delete a restriction endonuclease site which can be easily detected.

Alternatively, the difference in  $T_m$  (point of 50% irreversible melting) of hybrids formed between the oligonucleotide primer and either the original sequence of the mutant sequence can form the basis of a hybridization screening  
5 procedure (e.g., Zoller and Smith, 1982).

In order to increase the yield of mutants, the pool of transformed cells can be used to prepare a mixed population of single stranded (ss) DNA molecules. These can then be used as a template for a further round of annealing/transcription under  
10 conditions favouring the annealing of the oligonucleotide to the mutant sequence rather than the original sequence.

The DNA sequences illustrated in Charts 1 to 8 illustrate one preferred example of a DNA sequence that will code for the synthesis of the modified interferon amino acid sequence  
15 illustrated on the same chart. The genetic code (Lehninger, Biochemistry, Worth Publishing p. 962) will allow the design and synthesis (Nucleic Acids Research 11 477, 1983) of additional DNA sequences which also code for the amino acid sequences described in Charts 1 to 8. Such additional  
20 sequences could also be utilized in the plasmids pJA1, pJA2 and pJA3 to produce the modified-interferons of the present invention.

1. ConstructionsTABLE 1

## NOMENCLATURE FOR CONSTRUCTIONS

5	<u>CONSTRUCTION</u>	<u>TRIVIAL NAME</u>	<u>PRODUCT</u>
			<u>IDENTIFICATION</u>
	I	M13-1RB-00	HuIFN- $\beta$
	II	M13-4AB-00	HuIFN- $\beta$
	III	mJA1	HuIFN-X802
	IV	mJA2	HuIFN-X803
10	V	mJA3	HuIFN-X804
	VI	pJA1	HuIFN-X802
	VII	pJA2	HuIFN-X803
	VIII	pJA3	HuIFN-X804

M13 is a bacteriophage, a bacterial virus, more  
 particularly, a so-called "filamentous phage", which infects  
 male Escherichia coli (E.coli) cells. Unlike many  
 bacteriophages, the DNA found in the virus or phage particle is  
 single stranded (ss DNA). Upon infection of an E.coli cell,  
 the ss DNA is converted to the corresponding double stranded  
 form (ds DNA) and amplified, under suitable conditions of  
 growth, to, for example, from 200 to 300 copies per cell. The  
 phage-infected cells do not lyse releasing the new phage  
 particles, but continue to grow and divide, although at a  
 reduced rate. This may be seen on agar plates as a lighter



area, a "plaque", of slower-growing cells against a background of uninfected cells.

The ds DNA is also termed the "replicative form" (RF). This is comparable to a bacterial plasmid and may be used as a cloning and expression vector. The RF is central to the phage multiplication process. It is the template for ss DNA synthesis and for expression via protein biosynthesis of M13 phage genetic information. The former process produces phage particles and the latter allows the synthesis of phage proteins which are required to direct infected cells to produce further phage particles.

Wild type M13 has previously been modified for use as a cloning vehicle by the insertion of a fragment of E.coli DNA containing the lactose operon control region (lac promoter) and coding information for an active  $\beta$ -galactosidase ( $\beta$ -gal) in a non-essential region of the phage DNA. When the lac promoter is active, the expression of  $\beta$ -gal occurs and this is detected by a simple blue colour reaction in the infected cells. However, the cloning of DNA fragments generally results in the interruption of the lac Z gene and hence in colourless plaques due to the failure of  $\beta$ -gal expression. Thus, recombinants may be detected visually.

For example, U.K. Patent No. 1,588,572, which is directed to the production of filamentous hybrid phages, is representative of the stage of the art outlined above.

- 17 -

The known system allows the cloning of different-sized fragments of foreign DNA and the identification of recombinant clones without relying on the use of antibiotic resistance genes. Also, it allows the possibility of easily purifying  
5 phage ss DNA for use in recombinant characterisation and DNA sequencing, and site directed mutagenesis.

The known bacteriophage M13 mp 701 may be regarded as a starting point for the present invention. This vector, which is freely available, was constructed from M13mp7, which is  
10 commercially available, by known methods. Similarly, M13mp7 was in turn constructed from M13mp2. Reference may be made in this connection to, for example, Messing, J., et al., Proc.Natl.Acad.Sci.U.S.A., 74, 3642. (1977).

The plasmids pJA1, pJA2 and pJA3 in E coli K12 strain  
15 HB101 have the following ATCC designations; 39520, 39521 and 39522.

The American Type Culture Collection (ATCC) is located at 12301 Parklawn Drive, Rockville, MD 20852 USA.

#### Example 1

20 Construction I (M13-IRB-00, HuIFN-8)

Recloning of a human fibroblast interferon gene plus trp promoter to give M13-1RB-00 (see Fig. 2):

A 1172 bp DNA fragment containing the trp promoter followed by the mature HuIFN- $\beta_1$  gene bounded on the left by an

5 EcoRI site and on the right by a Bam HI site (GB Patent Application No. 2,068,970) was recloned between the EcoRI and Bam HI sites of phage M13 mp 701 as follows:

The joining of the EcoRI-Bam HI fragment containing the HuIFN- $\beta_1$  gene to the EcoRI-Bam HI digested M13 mp 701

10 vector was performed in an incubation of 50  $\mu$ l containing: 0.25  $\mu$ g vector; 0.9  $\mu$ g EcoRI-Bam HI cut p1/24 (GB P). Approximately 8% of plaques on each plate were colourless, indicating the presence of recombinant phages (1RB-00). Recombinants were firmly identified by size and by nucleotide  
15 sequence analysis, also by expression of antiviral activity.

To prepare sufficient ss DNA for nucleotide sequence analysis, colourless plaques were picked and added to 2.5 ml YT medium containing 25  $\mu$ l of a dense, overnight culture of  
20 E.coli K12 JM101. Phage was grown by aeration for 5 hours at 37°C and the ss DNA purified by known methods, (see, for example, Sanger, F., et al, (J. Mol. Biol., 143, 161, 1980). The ss DNA was used as the template for dideoxy sequencing, (see, for example, Sanger, F., et al, Proc. Natl. Acad. Sci. U.S.A., 74, 5463, 1977). For example, the presence and

sequence of the trp promoter and the presence of the HuIFN- $\beta$  gene was established with an oligonucleotide primer, IFIA (GB Patent Application No. 2,068,970) which is known to prime in the HuIFN- $\beta$  coding region.

5    Example 2

Construction II. (M13-4AB-00, HuIFN- $\beta$ )

Deletion of the lac promoter from M13-1RB-00(I) to give a recombinant capable of expressing mature HuIFN- $\beta$  under the control of only the trp promoter, II = M13-4AB-00) (See Fig.  
10    3).

Construction of this clone was achieved by excision of a 406 bp Ava I-EcoRI fragment as follows: Ava I-EcoRI double digestion was effected in 100  $\mu$ l containing: 10  $\mu$ g ds DNA prepared from construction I, (see, for example, Birnboim, 15    H.C., and Doly, J., Nucl. Acids Res., 7, 1513, 1979), 6 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 6 mM 2-mercaptoethanol, 30 mM NaCl, 15 units Ava I and 15 units EcoRI for 90 minutes at 37°C. The DNA was precipitated by the addition of 20  $\mu$ g tRNA; 0.3 M NaAc, pH 4.5 to 0.3 M final concentration and 0.3  
20    ml ethanol for 10 minutes at -70°C. Repair of protruding 5'-ends, with DNA polymerase, was then done in a 50  $\mu$ l final volume.

- 20 -

To "fill-in" protruding 5'-ends, the DNA fragments were repaired in vitro with DNA polymerase I (Klenow fragment) in a 50  $\mu$ l reaction containing 1 $\mu$ g DNA in 10 mM NaCl, 50 mM Tris-HCl, pH 7.8, 10 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 0.2 mM each deoxynucleoside 5'-triphosphate, 20  $\mu$ g/ml. bovine serum albumin, 0.2 mM each deoxynucleoside 5'-triphosphate, and 1 unit of Klenow enzyme for 20 minutes at 14°C in a volume of 50  $\mu$ l, then for 10 minutes at 65°C.

The repaired DNA was self-ligated in a 50 $\mu$ l incubation containing 5  $\mu$ l of the above incubation (equivalent to 0.1  $\mu$ g DNA), 66 mM Tris-HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 20mM DTT, 1mM ATP, and 4.5 units T4 DNA ligase for 17 hours at 25°C. Uptake of DNA into CaCl<sub>2</sub>-treated E.coli K12 JM101 and plating out of cells for plaques was performed using standard methods.

The identity of the new clone (M13-4AB-00) was confirmed by Tag I restriction analysis.

### Example 3

#### Construction III (mJA1), HuIFN-X802)

Alteration of Human Interferon $\beta$  (HuIFN $\beta$ ) amino acid 3 (tyrosine) to cysteine, to give mJA1. (See Figs. 3 and 4 and Chart 1)

- 21 -

A tetradecamer of the sequence 5' OH-AGT TGC AGC TCA TG-OH (Fig. 4) was constructed using standard phosphotriester chemistry; Reference: Nucleic Acids Research 11 477 (1983). This sequence is complementary to the sequence 5'C ATG ACC TAC AAC T which consists of the nucleotide preceding the initiator ATG followed by the first 13 nucleotides of the coding sequence of Human IFN- $\beta$  - (GB Patent Application No. 2,068,970) with the exception that the 6th nucleotide of the primer, numbered from the 5' end corresponds to the complement of the desired mutation. This mutation will change the nucleotide A at position 8 of the coding sequence to a G.

Specific priming of the synthetic oligonucleotide to the region which was to be mutated was observed by the following procedure. First the oligonucleotide was labelled with [ $^{32}\text{P}$ ] at the 5' terminus. 10 pmole of oligonucleotide was incubated in a total volume of 50  $\mu\text{l}$  consisting of 50 mM Tris-HCl pH 7.6, 10 mM  $\text{MgCl}_2$ , 0.1 mM EDTA (ethylene diamine tetra acetic acid), 10 mM dithiothreitol, 0.1 Mm spermidine, 50  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (5000 Ci/mmole, Amersham) and 5 units of polynucleotide kinase. The reaction mix was incubated at 37°C for 60 minutes, followed by 65°C for 5 minutes. The oligonucleotide was separated from unincorporated [ $\gamma$ - $^{32}\text{P}$ ] ATP by differential elution from a column of Whatman DE52 ion exchange resin (0.3 ml bed volume). The oligonucleotide was eluted in 0.5 M NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 20  $\mu\text{g}$  E.coli tRNA was added and the oligonucleotide was precipitated

at -70°C after the addition of three volumes of ethanol. The oligonucleotide was redissolved in 8 µl of 10 mM Tris HCl pH 7.5, 1 mM EDTA.

5 pmoles of [<sup>32</sup>P] oligonucleotide were annealed to 0.5 pmole of M13-4AB-00 in a total volume of 5 µl of 10 mM NaCl, 40 mM Tris-HCl pH 7.5, 20 mM MgCl<sub>2</sub>, 2 mM 2-mercaptoethanol, by heating at 80°C for 5 minutes followed by incubation at 20°C for 1 hour.

The samples were adjusted to 67.5 mM NaCl, 45 mM Tris-HCL pH 7.5, 25 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 83 uM each of dATP, dCTP, dGTP, dTTP and 0.5 unit of the 'Klenow' fragment of DNA Polymerase I (BRL Inc.) in a final volume of 15 µl. The samples were incubated at 25°C for 4 hours. The samples were diluted eight fold with 10 mM Tris-HCL pH 7.5, 1 mM EDTA, heated to 65°C for 5 minutes, adjusted to 0.3 M Na acetate pH 4.6 and finally the nucleic acid was precipitated with three volumes of ethanol at -70°C for 15 minutes. In order to identify specific priming products the samples were digested with the enzyme EcoRI and the products separated by electrophoresis through a 7M Urea, 6% Acrylamide, 0.3% Bis (NN'-methylene bisacrylamide) gel prepared in 135 mM Tris-HCL pH 8.8, 45 mM Na borate, 1 mM EDTA.

A specific transcription product of 150 bases was identified which corresponds to the instance between the

- 23 -

upstream E.coli site in 4AB-00 and the 5' end of the oligonucleotide, given specific priming.

Formation of complete closed circular molecules was achieved as follows: The oligonucleotide was phosphorylated as described earlier except that ATP at 1mM final concentration  
5 replaced the [ $\gamma$ - $^{32}$ P] ATP. The oligonucleotide was annealed as described earlier except that a primer to template ratio of 100 was used (50 pmole oligonucleotide per 0.5 pmole template).

The transcription and ligation of the product was performed  
10 as follows: 10 $\mu$ l of annealed primer-template, containing 0.5 pmole of template and 50 pmole of primer in 100 mM NaCl, 40mM tris-HCl pH 7.5, 20mM MgCl<sub>2</sub>, 2mM 2-mercaptoethanol was adjusted to a volume of 20 $\mu$ l containing 20 $\mu$ Ci [ $\alpha$ - $^{32}$ P] dATP (2000ci/mmole) 250 $\mu$ M each of dATP, dCTP, dGTP, dTTP, 0.8  
15 unit of Klenow DNA polymerase I, 2 units T<sub>4</sub> ligase (BRL Inc.) in 67.5mM NaCl, 45mM Tris pH 7.5, 25mM MgCl<sub>2</sub>, 1mM 2-mercaptoethanol. After 30 minutes at 22°C dATP was added to 250 $\mu$ M and a further 0.8 unit of Klenow DNA polymerase was added. The mixture was incubated a further 2.5 hours at 22°C.  
20 The DNA was adjusted to 0.3M Na acetate pH 4.6, and precipitated with three volumes of ethanol at -20°C overnight (16 hrs).

Incomplete transcripts were digested with S1 nuclease as follows. The DNA was incubated for 30 minutes at 25°C in a



solution of 300mM NaCl, 5mM ZnCl<sub>2</sub>, 30mM Na acetate pH 4.5 at an initial template concentration of 1nM in a final volume of 250µl. S1 nuclease was added at the rate of 1 unit per 0.01 pmole of initial template.

5        The products were added directly to 0.4 mls of competent E.coli JM101 at 0°C. After 40 minutes the cells were heat shocked for 2 minutes at 42°C then diluted into 20 ml of YT broth (8g tryptone, 5g yeast extract, 5g NaCl per litre) at 37°C. The cells were grown at 37°C for 16 hours. The cells  
10       were pelleted by centrifugation and phage in the supernatant were stored in 60% glycerol at -20°C.

Dilutions of phage were plated on indicator cells and plates containing 2-400 plaques were used to make  
nitrocellulose replicates for hybridization, using the [<sup>32</sup>P]  
15       phosphorylated oligonucleotide as probe in a manner similar to that of Benton and Davis (Science 196, 180, 1977). Filters were prewashed in 6 x SSC at 40°C for 3 hrs and hybridized using 300µl of a solution of 6 x SSC/10 x Denhardts/0.1% SDS containing primer at 400pM for 16 hrs at 40°C, under paraffin  
20       oil [1 x SSC = 0.15M. NaCl; 0.015M Na citrate, pH 7.2. 10 x Denhardts = 0.2% Bovine Serum Albumin (BSA); 0.2% polyvinyl pyrrolidone (PVP); 0.2% Ficoll]. Filters were washed in 6 x SSC/0.1% SDS at 15°C, with six changes of five minutes each.

- 25 -

Fifty plaques which showed hybridization above background were picked into 50µl each of LTB (10mM Tris-HCl pH 7.5, 20mM NaCl, 1mM EDTA). Cellular debris was spun out and 1µl of supernatant for each plaque was spotted onto a lawn of E.coli JM101 which had been allowed to grow for 60 minutes at 37°C. After overnight growth large plaques were formed on the lawn of E.coli JM101. Nitrocellulose replicates were again taken and hybridised as above. Nineteen of the 50 plaques showed hybridization above control levels. These were rescreened exactly as described above. Six were taken for further analysis by DNA sequencing. As the desired change was A to G, the coding strand was analysed by 5'end-labelling followed by the G reactions of the Maxam and Gilbert chemical degradation sequencing technique (Maxam and Gilbert, Methods in Enzymology Vol 65 (1), p 499, 1980) while the non-coding strand was analysed by 3'end-labelling followed by the 'C' reaction of the Maxam and Gilbert technique. Thus mutants would exhibit an extra G in the coding strand and an extra C in the non-coding strand compared to parallel reactions on the parent M13-4AB-00. The DNAs were therefore digested with either Hind III to produce staggered ends for 3' labelling, or Hpa I to produce a blunt end for 5' labelling.

After labelling the fragments were digested with Bgl II to separate the labelled ends. The required fragments were isolated by electrophoresis on a 10% Acrylamide/0.33% bis-acrylamide gel in 135mM Tris-HCl pH 8.8, 45mM Na borate,

- 26 -

1mM EDTA. The fragments were electroeluted and concentrated on a 0.3ml Whatman DE52 ion exchange resin column prior to DNA sequencing. All six clones showed the desired change. Two of the clones were picked and tested to show that the gene product  
5 was still antivirally active.

Interferon anti-viral assays were performed on extracts of M13 infected or plasmid transformed cells as follows: Fifty 200ml. cultures in tryptophan-free minimal medium plus glucose were harvested at an optical density (600 nm) of 0.6-0.9 by  
10 centrifugation at 10,000 rpm for 10 minutes. The cells were then frozen at -70°C, thawed in the presence of 2.5-5.0ml of 15% (w/v) sucrose; 50 mM Tris-HCl, pH 8.0; 0.1% (w/v) human serum albumin and 2.5 mg lysozyme, then incubated at 20°C for 15 minutes with shaking. The cell debris was removed by  
15 centrifugation at 15,000 rpm for 20 minutes and the supernatant was further clarified and sterilised by filtration through a 0.22 um pore diameter nitrocellulose filter. Finally the extract was assayed for anti-viral activity by monitoring the protection conferred on Vero (African green monkey) cells  
20 against the cpe (cytopathic effect) of EMC (Encephalomyocarditis) virus infection in an in vitro microplate assay system (see, for example, Dahl, H., and Degre, M., Acta. Path. Microbiol. Scan., 1380, 863 1972).

Similar levels of antiviral activity to that shown by the  
25 parent were observed. Single-stranded phage DNA was isolated

from one clone by established procedures, to act as template for construction IV.

Example 4

Construction IV (mJA2, HuIFN-X803)

5       Alteration of mutant interferon  $\beta$  (mJA1) at amino acid 101 (valine) to cysteine, to give mJA2. (See Figures 3 and 5 and Chart 2).

10       An octadecamer of sequence 5' OH-CTTCCAGGCATGTCTTCA-OH 3' (Fig. 5) was constructed using phosphotriester chemistry, as for Construction III. This sequence is complementary to the sequence 5'TGAAGACAGTCCTGGAAG.3' which comprises nucleotides 294 to 310 of the IFN- $\beta_1$  coding sequence (see published GB Patent Application No. 2,068,970) with the exception that the 9th and 10th nucleotides of the oligonucleotide, numbered from 15 the 5' end, correspond to the complement of the desired mutations. Thus the desired changes will be from G at nucleotide 301 of the coding sequence to T, and from T at nucleotide 302 to G.

20       Specific priming was demonstrated exactly as for mJA1. The specific product was identified as a 348 base fragment after digestion of the transcription products with Hpa I and electrophoresis as previously described.

Formation of closed circular molecules, ligation, transfection were as described from Example 3. Phage DNA was prepared from the total pool of transformants and used as a template for reannealing of the primer, transcription, ligation,  $S_1$  nuclease treatment, and transfection as described, except that in the  $S_1$  reaction the conditions were changed to encourage digestion at the mismatch between parent template and primer, so enriching for mutant closed circular molecules in the population. The reaction therefore was performed in 300  $\mu$ l of 100 mM NaCl, 30mM Na acetate Ph 4.5, 5mM Zncl and containing 0.8 pmole of initial template and 2 units of  $S_1$  nuclease.

Phage DNA was prepared from the total pool of transformants and the enrichment stage was repeated again exactly as above. 50 plaques from the second round of enrichment were grown in 1ml (YT medium). 2 $\mu$ l of each suspension was spotted directly onto nitrocellulose and hybridised as above. Three positive plaques and two negative plaques were rescreened. Phage DNA was prepared and concentrated by polyethylene glycol (PEG) precipitation. Phage were resuspended in 50 $\mu$ l of LTB, representing a 300 fold concentration. 2 $\mu$ l of each suspension was spotted onto nitrocellulose and hybridised with [ $^{32}$ P] phosphorylated primer as described above. Hybridization confirmed the three positive plaques. The presence of the desired change was confirmed by DNA sequencing (Maxam and Gilbert). Single stranded phage DNA was prepared by

- 29 -

established procedures. A short oligomer complementary to the sequence coding for amino acids 114 to 117 of the  $\beta$  sequence was prepared and phosphorylated using [ $\gamma$ - $^{32}$ P] ATP as described previously. This was annealed to the phage DNA as described and short transcripts produced which were cleaved with Pst 1. A band of 213 b. corresponding to the specific priming product was isolated on a 7M urea, 8% Acrylamide 0.2% Bis-acrylamide 135mM Tris-HCl pH 8.8, 45mM Na borate, 1mM EDTA gel. The fragment was electroeluted and the DNA sequenced using the Maxam and Gilbert technique. The DNA sequence confirmed the desired changes (Fig. 5, 6). One clone was picked and tested to show that the gene product was still antivirally active as described earlier. This clone was used as a template for construction V.

#### 15 Example 5

##### Construction V (mJA3, HuIFN-X804)

Alteration of mutant human interferon  $\beta$  (mJA2) at amino acid 17 (cysteine) to serine to give mJA3 (See Fig. 3 and 6 and Chart 3).

20 A hexadecamer of sequence 5'OH-CTGACTCTGAAAATTG3' (See Fig. 6) was constructed using phosphotriester chemistry, as for Construction III and IV. This sequence is complementary to the sequence 5'CAATTTTCAGTGTCTG3' which comprises nucleotides 39 to 54 inclusive of the IFN- $\beta$  coding sequence (see published GB

Patent Application No. 2,068,970) with the exception that the 6th nucleotide of the oligonucleotide, numbered from the 5'end,, corresponds to the complement of the desired mutation. Thus the desired change will be from T to A at nucleotide 49 of the coding sequence.

Specific priming was demonstrated as for Example 3 and 4. The specific product was identified as a 187 base fragment after digestion of the transcription products with EcoRI and electrophoresis as previously described.

Formation of closed circular molecules and ligation was as described for constructions III and IV. Closed circular molecules were separated from incomplete products by electrophoresis through 1% low melting temperature agarose in 67.5 mM Tris-HCl pH8.8, 22.5mM Na borate, 0.5mM EDTA, 1µg/ml ethidium bromide without prior  $S_1$  nuclease treatment. The region corresponding to closed circular double stranded full length molecules was visualised under long wave (366nm) transillumination, cut from the gel and melted at 60°C for 5 minutes. A volume corresponding to 0.3 pmole of double stranded product was used to transfect E.coli JM101 by established procedures. The transfected cells were plated out in top agar directly. After overnight growth, nitrocellulose replicates were taken and hybridized with [ $^{32}$ P] phosphorylated primer as described. The hybridization temperature was 42°C for 36 hours. E.coli DNA (10 µg/ml,

heat denatured) was included in the prehybridization solution. Finally, the filters were washed at 44°C in 6 x SSC.

The 24 plaques which showed a signal above background were picked and grown for 6 hours in 1ml YT broth containing 25µl of log phase E.coli JM101. The cells were removed by centrifugation and the phage in solution were concentrated 80 fold by PEG precipitation, to a final volume of 10µl. 4µl of each was spotted onto nitrocellulose and hybridized using the [<sup>32</sup>P] primer. About 50% of the spots showed a signal greater than the background level. Four of the positive phage were further plaque purified. Phage was isolated from distinct, positively hybridizing plaques and the dsDNA replicative form (RF) prepared. The presence of the desired change was inferred by the appearance of a novel Hinf I site. The desired change, T to A, introduces the sequence 5' CAGTC which is a recognition sequence from the enzyme Hinf I. The presence of the site causes a Hinf I fragment of 197 base pairs in the parent RF to be cleaved to two molecules of 169 and 28 base pairs in the mutant RF.

Thus 5µg of RF was digested with 12.5 units of Hinf I in a total volume of 500µl of 6mM Tris-HCl pH 7.5, 6mM MgCl<sub>2</sub>, 6mM 2-mercaptoethanol, 50mM NaCl. for 16 hours at 37°C. The fragments were labelled at their 3' ends with [<sup>32</sup>cP] dATP as the restriction enzyme cleaves between the G and A of the recognition site. Thus the reaction consisted of 0.4 pmole of



- 32 -

DNA, 20 $\mu$ Ci [ $\alpha$ -<sup>32</sup>P] dATP (2000Ci/mmol) and 2 units of Klenow DNA polymerase I in 50 $\mu$ l of 6mM Tris-HCl pH 7.5, 50mM NaCl, 6mM MgCl<sub>2</sub>, 7mM mercaptoethanol, at 25°C for 60 minutes. The fragments were separated on 10% acylamide, 0.33% Bis-acrylamide, in 135mM Tris-HCl pH 8.8, 45mM Na borate, 1mM EDTA. All of four plaques analysed showed the desired restriction pattern indicating the desired mutation had been induced.

#### Example 6

#### 10 Constructions VI, VII, VIII

VI: pJA1, HuIFN-X802

VII: pJA2, HuIFN-X803

VIII: pJA3, HuIFN-X804

Subcloning of mutant  $\beta$  constructs into pMN39-1. (See  
15 Fig. 7).

Plasmid pMN39-1 consists of a deletion of 434 bp between the Bgl II and Bam HI site of plasmid p1-24. pMN39-1 therefore contains the natural HuIFN- $\beta$  gene under trp attenuator minus control. The trp control region and 161  
20 amino acids of the IFN- $\beta$ , gene are present on a 621 bp EcoRI/BstEII fragment. This fragment can be removed and replaced by the analogous fragments from mJA1, mJA2 or mJA3 to produce pJA1, pJA2, pJA3 respectively. These constructs would

thus represent the mutant HuIFN- $\beta$  genes under trp control on a high copy number plasmid also coding for the  $\beta$ -lactamase gene so allowing selection by conferring ampicillin resistance on a transformed E.coli cell.

5           In order to achieve the subcloning RF from mJA1, mJA2, mJA3, and closed circular plasmid pMN39-1 were digested with the enzymes EcoRI and BstEII. One pmole each of mJA1, mJA2 and mJA3 were digested with 10 units of BstEII for 16 hours at 37°C in a total volume for each reaction of 250 $\mu$ l. 2 pmole  
10 of pMN39-1 was digested with 10 units of BstEII for 16 hours at 37°C in a total volume of 250  $\mu$ l.

The DNAs were precipitated and redigested with 20 units each of EcoRI for 16 hours at 37°C in a total volume of 250  $\mu$ l. The digestion products were precipitated and redissolved  
15 in 20  $\mu$ l of 10 mM Tris-HCl pH 7.5, 1 mM EDTA. The products of the digestion of pMN39-1 were resolved on a 0.8% low melting temperature agarose gel in 67.5 mM Tris-HCl pH 8.8, 22.5 mM Na borate, 0.5 mM EDTA containing 1  $\mu$ g/ml ethidium bromide. The 3303 bp EcoRI/BstEII fragment was cut from the gel and melted  
20 at 60°C.

The products of the digestion of mJA1, mJA2, mJA3 were resolved as above except that the agarose concentration was 2%. The 621 bp EcoRI/BstEII fragment from each digest was cut from the gel and melted at 60°C.

- 34 -

A volume calculated to contain 0.17 pmole of the 3.3 kb pMN 39-1 fragment was mixed with a volume calculated to contain 0.4 pmole of the 0.62 kb fragment for each of mJA1, mJA2, mJA3. The fragments were ligated with 1 unit of T4 ligase in a total  
5 volume of 200  $\mu$ l of 65 mM Tris-HCl pH 7.5, 5mM Mg Cl<sub>2</sub>, 20 mM dithiothreitol, 1 mM ATP for 24 hours at 20°C.

Fifty  $\mu$ l of each ligation mix was used to transform 0.3 ml of competent E.coli K12 HB101 cells by established procedures.

10 Several transformants for each ligation were grown and plasmid DNA prepared by the method of Birnboim and Doly (Nucleic Acids Res. 7 1573, 1979). The plasmid DNA was digested with HinfI and compared against pMN39-1 as described for the analysis of Construction V. For pJA1 and pJA2 the  
15 digestion pattern was shown to be identical to pMN39-1. The HinfI digest of pJA3 differed from that of pMN39-1 in that the 197 bp band was not present but was replaced by bands of 169 and 28 bp, as expected.

Example 7Antiviral properties of modified IFN- $\beta$ 

Antiviral assays were performed as described in the description of the construction of mJA1 (Construction III).

5 In the cases of constructions VI - VIII the medium contained ampicillin at 100 ug/ml.

Yields of interferon were obtained for constructions I-IV as indicators that the constructs still retained biological activity (Table 2). The A600 was 0.4 when induced and 1.0 when  
10 harvested. In all cases expression from the trp promoter could be detected in inducing conditions. However, yields ranged from  $6 \times 10^3$  to  $5.3 \times 10^4$  IU./L for mJA3 (Construction II),  $3.7 \times 10^3$  to  $7.9 \times 10^4$  IU/L for mJA1 (Construction III), and  $1.45 \times 10^3$  to  $2 \times 10^5$  IU/L for mJA2 (Construction IV).

15

TABLE 2

Experiment No.	Construct (Trivial Name)	Antiviral Titre	
		IU/L	Mean IU/L
1.	pMN39-1	$2.3 \times 10^7$	$2.3 \times 10^7$
	VI pJA1	$4.8 \times 10^5$	$6.2 \times 10^5$
20	VI pJA1	$7.6 \times 10^5$	
	VII pJA2	$1.14 \times 10^6$	$1.2 \times 10^6$
	VII pJA2	$1.33 \times 10^6$	

- 36 -

2.		pMN39-1	$3.5 \cdot 10^7$
	VII	pJA2	$6.65 \times 10^6$
3.		pMN39-1	$6.35 \times 10^6$
	VII	pJA2	$3 \times 10^5$
5	VIII	pJA3	$2.1 \times 10^6$

The antiviral titres of the plasmid constructs VI to VIII have been investigated. Experiments (Table 2) indicated a reduction in the titre compared to the parental plasmid pMN39-1. In particular the A to G alteration from pMN39-1 to pJA1 results in an apparent drop in the titre of more than one order of magnitude.

#### SUMMARY OF TABLE 2

	<u>Construct (Trivial Name)</u>	<u>Antiviral Titre (IU/L)</u>
	pMN39-1	$2.14 \times 10^7$
15	pJA1	$6.2 \times 10^5$
	pJA2	$2.35 \times 10^6$
	pJA3	$2.1 \times 10^6$

Data from heterologous cell assays performed with crude lysate material indicate no alteration in activity between the pMN39-1 product and that of pJA1 and pJA2.

Determination of -SH groups in proteins

The presence of sulfhydryl groups S in the modified interferons is determined by spectrophotometric titration with p-mercuribenzoate by the method of Boyer (1954). Boyer P.D. 5 (1954), J.Amer.Chem. Soc. 76,4331. This method is performed in, for example, BM urea or 0.5 to 1% sodium dodecyl sulphate to "unmask" buried -SH groups. Other methods based on alkylation reactions are appropriate, e.g. use of N-ethylmaleimide (NEM). Gregory, J.D. (1955), J.Amer.Chem.Soc. 10 77,3922. One can also use methods based on reactions with disulphides e.g. 5,5'-dithiobis (2-nitrobenzoate) by the method of Ellman (1959). Arch.Biochem. and Biophys. 82,70, yielding a chromophore which may be quantitated spectrophotometrically.

Determination of -S-S- groups in proteins

15 The methods used for determining the sulfhydryl (-SH) content of proteins are applicable to the determination of the (-S-S-) disulfide (-S-S-) content, after first reducing the -S-S- bridges with a reducing agent. The number of -S-S- bonds may then be deduced from knowledge of the number of -SH groups in 20 the non-reduced protein relative to the total -SH content after reduction. For example, a method was developed by Cavallini et al (1966), Nature 212,294 where -S-S- groups were reduced with borohydride, and the number of -SH groups formed determined by using the method of Ellman (ibid).

Determination of the position of disulphide bonds  
in proteins

The formation of strongly acidic sulphonic groups by the oxidative cleavage of S-S bonds sharply changes the electrophoretic mobility of peptides containing cysteine. 5 Brown and Hartley (1963,1966) (1963,Biochem.J., 89,59P) (1966,Biochem.J., 101,214) developed a method for locating S-S bridges in proteins using "diagonal electrophoresis" of peptides on paper. An enzymic hydrosylate is subjected to 10 electrophoresis at pH6.5. After drying, a strip is cut out from the electrophore- gram and is sewn onto a new sheet of paper. A second electrophoresis is then carried out at pH6.5 at a right angle to the direction of the first electrophoresis. As a result, the peptides are arranged, 15 according to their mobilities, along a diagonal line. If the strip of paper cut out after the first electrophoresis is exposed to vapours of performic acid, the peptides containing cysteic acid formed by this treatment are located off the diagonal. By this method, one easily identifies the peptides 20 that are linked in a protein by S-S bridges.

Such peptides are eluted from the paper and subjected to amino-acid analysis and/or peptide sequencing and thus the location of peptide bonds in the protein determined.

Example 8Construction of IFNX815, IFNX816, IFNX817, IFNX818, and IFNX457

The DNA nucleotide sequence and amino acid sequence of the modified-interferons IFNX815, 816, 817, 818, and 457 are shown in charts 4-8. These DNA sequences are constructed from the plasmids pJA1, pJA2, and pJA3 utilizing the methods of Examples 3, 4, 5, and 6. The modified interferons are isolated using the methods of Example 9. Alternatively, an entire DNA sequence specified by charts 4-8 is synthesized using the standard genetic code indicating triplet codons and standard phosphotriester chemistry of Examples 3, 4, and 5. Plasmids pMN39-1, pJA1, pJA2, or pJA3 are then utilized to produce the plasmids utilizing the method of Example 6.

Example 915 Interferon Extraction and Purification

The interferons can be isolated from the producing cell by use of the following procedure:

Step 1. Centrifugation of broken cell preparation.

Step 2. Resuspend the pellet in 50mM Tris-Cl pH 8.0 with three-fold w/w/ excess of SDS over protein. Add DTT



to 100mM and warm to 95°. Hold at 95° for five minutes.

Step 3. Centrifuge to obtain a clarified extract.

Step 4. Gel filter on an Aca44 column (4.4 X 60 cm) with 50mM  
5 Tris-Cl pH 8.0, 10mM DTT, 0.5% SDS as eluent,  
40ml/hr. This column size will allow the proceeds of  
a nominal 10L fermentation (at the current cell  
density) to be processed in one batch.

#### Parenteral Administration

10 The modified interferons of the present invention can be  
formulated according to methods well known for pharmaceutical  
compositions, wherein the active interferon polypeptide is  
combined in admixture with a pharmaceutically acceptable  
carrier vehicle, such as albumin. Remington's Pharmaceutical  
15 Sciences by E. W. Martin, hereby incorporated by reference,  
describes composition and formulations suitable for delivery of  
the interferons on the present invention.

Pharmaceutical compositions of the present invention will  
contain an effective amount of the interferon protein together  
20 with a suitable carrier allowing therapeutic administration.  
One mode of administration is parenteral, another mode of  
administration is nasal spray.

Administration of the interferon composition is indicated for patients requiring antitumor cell growth or immune modulation, or antiviral treatment. Dosage and dose rate may parallel those now in use in clinical trials of approximately  $10^5$  to  $10^8$  units daily. Dosages significantly below or above these levels may be indicated in long term administration or during acute short term treatment. It is anticipated that a preferred dosage rate is  $10^6$ - $10^7$  units daily for parenteral administration.

CLAIMS

1. A modified-interferon molecule comprising the amino acid sequence of a first interferon with one or more cysteine deletions or substitutions at said first interferon sequence positions corresponding to a second interferon's cysteine positions.  
5
2. The modified-interferon of Claim 1 wherein the cysteine deletions or substitutions permit the formation of one or more disulfide linkages at positions corresponding to the disulfide linkages in a second interferon.
- 10 3. The modified-interferon of Claim 2 wherein the first interferon is human beta interferon and the second interferon is a human alpha interferon.
4. The modified-interferon of Claim 2 wherein the first interferon is human alpha interferon and the second  
15 interferon is human beta interferon.
5. The modified-interferon of Claim 2 wherein the first interferon is human beta interferon and the second interferon is human gamma interferon.

- 43 -

6. The modified interferon of Claim 2 wherein the first interferon is human gamma interferon and the second interferon is human beta interferon.
7. The modified-interferon of Claim 2 wherein the first  
5 interferon is a human alpha interferon and the second interferon is a human gamma interferon.
8. The modified-interferon of Claim 2 wherein the first interferon is a human gamma interferon and the second interferon is a human alpha interferon.
- 10 9. The modified-interferon of Claim 3 wherein one of the beta interferon amino acids 1 to 6 are replaced by a cysteine.
10. The modified-interferon of Claim 3 wherein one of the beta interferon amino acids 97 to 105 are replaced by a cysteine.
11. The modified-interferon of Claim 9 wherein one of the beta  
15 interferon amino acids 97 to 105 are replaced by a cysteine.
12. A modified-interferon of Claim 9 wherein the amino acid sequence comprises the sequence of IFNX 802.
13. A modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 803.

- 44 -

14. A modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 804.
15. The modified-interferon of Claim 9 wherein the amino acid sequence comprises the sequence of IFNX 815.
- 5 16. The modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 816.
17. The modified-interferon of Claim 9 wherein the amino acid sequence comprises the sequence of IFNX 817.
18. The modified-interferon of Claim 11 wherein the amino acid  
10 sequence comprises the sequence of IFNX 818.
19. The modified-interferon of Claim 11 wherein the amino acid sequence comprises the sequence of IFNX 457.
20. The modified-interferon of Claim 11 wherein a disulfide  
15 bond is formed between a Cysteine in positions 1 to 6 and a Cysteine in positions 97 to 105.
21. The modified-interferon of Claim 14 wherein a first disulfide bond is formed between a Cysteine 1 to 6 and a Cysteine 97 to 105 and a second disulfide bound is formed between Cysteine 31 and Cysteine 141.

- 45 -

22. A bacterial extract comprising greater than 90% pure polypeptide consisting essentially of the amino acid sequence of the hybrid or modified interferons according to any one of claims 1-21.
- 5 23. A pharmaceutical composition comprising a therapeutically effective amount of a modified interferon according to any one of claims 1-21, together with a pharmaceutically acceptable diluent.
24. A composition according to claim 23 suitable for parenteral administration.
- 10 25. A hybrid or modified interferon according to any one of claims 1-21 or a composition according to claim 23 or 24 for use in an anti-viral, anti-proliferative, anti-tumour, immunomodulatory or immunogenic method of treatment of the human or animal body.
- 15 26. A process for producing a modified interferon claimed in any one of claims 1-21 which process comprises causing a microorganism, transformed with a replicable microbial expression vehicle capable of expressing said modified interferon and recovering said modified interferon.
- 20 27. A process for producing microorganisms capable of expressing a modified interferon claimed in any one of claims 1-19 comprising: a) transforming a microorganism with a replicable microbial expression vehicle capable of expressing said modified interferon and b) fermenting the
- 25 transformed microorganism.

28. A method of stabilizing a modified interferon comprising the creation of new disulfide bonds.
29. A DNA polymer comprising a nucleotide sequence that encodes for the synthesis of the polypeptide IFNX802, IFNX803, IFNX804, IFNX815, IFNX816, IFNX817, IFNX818 or IFNX457.
30. A plasmid containing the DNA polymer of claim 29.
31. The plasmid of claim 30 comprising the plasmid pJA1 (ATCC 39520).
32. The plasmid of claim 30 comprising the plasmid pJA2 (ATCC 39521).
33. The plasmid of claim 30 comprising the plasmid pJA3 (ATCC 39522).

IFN $\beta$  802IFN $\beta$ [Tyr<sup>3</sup> -> Cys<sup>3</sup>]

5 10 15  
 MET-SER-CYS-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG AGC TGC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-VAL-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA  
 110 120 125  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 130 135 140  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 145 150 155  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 160 165 170  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA  
 ASN-TER-  
 AAC TGA

MSCNLLGFLQRSSNFQ CQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAALTIY  
 ENLQNI FAIFRQDSSSTGWN ETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSL  
 HLKRY YGRILHYLKAKEYSHCAWTIVRVEILRN FYFINRLTGYLRN<



IFN $\beta$  803IFN $\beta$ [Tyr<sup>3</sup> -> Cys<sup>3</sup>][Val<sup>101</sup> -> Cys<sup>101</sup>]

5 10 15  
 MET-SER-CYS-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG AGC TGC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT

20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC

35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG

50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT

65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT

80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT

95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA

110 120 125  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG

130 135 140  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC

145 150 155  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC

160 165 170  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-TER-  
 AAC TGA

MSCNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDPPEIKQLQQFQKEDAAALTIY  
 EMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTCLEEKLEKEDFTRGKLMSSL  
 HLKRYYYGRILHYLKAKEYSHCAWTIVRVEILRNIFYFINRLTGYLRN<

IFNX 804

IFN $\beta$ [Tyr<sup>3</sup> -> Cys<sup>3</sup>][Cys<sup>17</sup> -> Ser<sup>17</sup>][Val<sup>101</sup> -> Cys<sup>101</sup>]

MET-SER-CYS-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG AGC TGC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 GLN-SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG AGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA  
 ASN-TER-  
 AAC TGA

MSCNLLGFLQRSSNFQSQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQOQFKEDAALTIY  
 EMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTCLEEKLEKEDFTRGKLMSSL  
 HLKRYYGRIHLHYLKAKEYSHCAWTIVRVEILRNIFYFINRLTGYLNRK<

IPNX815

IPN- $\beta$  [ser<sup>2</sup>→cys<sup>2</sup>] [cys<sup>17</sup>→ser<sup>17</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT

20 25 30  
 GLN-SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG AGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC

35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG

50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT

65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT

80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT

95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-VAL-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA

110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG

125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC

140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC

155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
 AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQSQKL-LWQLNGRLEY-CLKDRMNFDI-PEEIKQLQRF-

60 70 80 90 100  
 QKEDAALTIY-EMLNIFAIF-RQDSSSTGWN-ETIVENLLAN-VYHQINKLKT-

110 120 130 140 150  
 VLEEKLEKED-FTRGKLMSSL-HLKRYYGRIL-HYLKAKKEYSH-CAWTIURVEI-

## IFNX816

IFN- $\beta$ [ser<sup>2</sup>→cys<sup>2</sup>][cys<sup>17</sup>→ser<sup>17</sup>][val<sup>181</sup>→cys<sup>181</sup>]

5 10 15  
MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT

20 25 30  
GLN-SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
CAG AGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC

35 40 45  
CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG

50 55 60  
GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT

65 70 75  
GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT

80 85 90  
SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT

95 100 105  
VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA

110 115 120  
LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG

125 130 135  
HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC

140 145 150  
LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC

155 160 165  
LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
AAC TGA

10 20 30 40 50  
MCYNLLGFLQ-RSSNFQSQKL-LWQLNGRLEY-CLKDRMNFDI-PEETKQLQKF-

60 70 80 90 100  
QKEDAALTIY-EMLNIFAIF-RQDSSSTGLN-ETIVENLLAN-VYHQINHLKT-

110 120 130 140 150  
CLEEKLEKED-FTRGKLMSSL-HLKRYYGRIL-HYLKAKEYSH-CAVTIURVEI-

160  
LRNFYFINRL-TGYLRN<

IPNX817

IFN- $\beta$ [ser<sup>2</sup>→cys<sup>2</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-VAL-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA GTC CTG GAA GAA AAA  
 110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
 AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQCQKL-LWQLNGRLEY-CLKDRMNFDI-PEEIKQLQKF-  
 60 70 80 90 100  
 QKEDAALTIY-EMLQNI FAIF-RQDSSSTGWN-ETIVENLLAN-VYHQINHLKT-  
 110 120 130 140 150  
 VLEEKLEKED-FTRGKLMSSL-HLKRYYGRIL-HYLKKEYSH-CAWTIVRVEI-  
 160

## IPNX818

IPN- $\beta$ [ser<sup>2</sup>->cys<sup>2</sup>][val<sup>181</sup>->cys<sup>181</sup>]

5 10 15  
 MET-CYS-TYR-ASN-LEU-LEU-GLY-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-  
 ATG TGT TAC AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT  
 .. 20 25 30  
 GLN-CYS-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-  
 CAG TGT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC  
 .. 35 40 45  
 CYS-LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-  
 TGC CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG  
 .. 50 55 60  
 GLN-LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-  
 CAG CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT  
 .. 65 70 75  
 GLU-MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-  
 GAG ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT  
 .. 80 85 90  
 SER-THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-  
 AGC ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT  
 .. 95 100 105  
 VAL-TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-  
 GTC TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA  
 .. 110 115 120  
 LEU-GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-  
 CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG  
 .. 125 130 135  
 HIS-LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-  
 CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC  
 .. 140 145 150  
 LYS-GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-  
 AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC  
 .. 155 160 165  
 LEU-ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-  
 CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA

ASN-\*\*\*-  
 AAC TGA

10 20 30 40 50  
 MCYNLLGFLQ-RSSNFQCQKL-LWQLNGRLEY-CLKDRMNFDI-PEEIKQLQGF-  
 60 70 80 90 100  
 QKEDAALTIY-EMLQNI FAIF-RQDSSSTGWN-ETIVENLLAN-VYHQINHLKT-  
 110 120 130 140 150  
 CLEEKLEKED-FTRGKLMSSL-HLKRYYGRIL-HYLKAKEYSH-CAWTIVRVEI-

IPN- $\beta$ [IPN- $\beta$ 2-7 $\rightarrow$ IPN- $\alpha$ 21-5][cys<sup>16</sup> $\rightarrow$ ser<sup>16</sup>][val<sup>108</sup> $\rightarrow$ cys<sup>108</sup>]

5	10	15
MET-CYS-ASP-LEU-PRO-GLN-PHE-LEU-GLN-ARG-SER-SER-ASN-PHE-GLN-		
ATG TGC GAC TTA CCA CAA TTC CTA CAA AGA AGC AGC AAT TTT CAG		
20	25	30
SER-GLN-LYS-LEU-LEU-TRP-GLN-LEU-ASN-GLY-ARG-LEU-GLU-TYR-CYS-		
TCT CAG AAG CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAT TGC		
35	40	45
LEU-LYS-ASP-ARG-MET-ASN-PHE-ASP-ILE-PRO-GLU-GLU-ILE-LYS-GLN-		
CTC AAG GAC AGG ATG AAC TTT GAC ATC CCT GAG GAG ATT AAG CAG		
50	55	60
LEU-GLN-GLN-PHE-GLN-LYS-GLU-ASP-ALA-ALA-LEU-THR-ILE-TYR-GLU-		
CTG CAG CAG TTC CAG AAG GAG GAC GCC GCA TTG ACC ATC TAT GAG		
65	70	75
MET-LEU-GLN-ASN-ILE-PHE-ALA-ILE-PHE-ARG-GLN-ASP-SER-SER-SER-		
ATG CTC CAG AAC ATC TTT GCT ATT TTC AGA CAA GAT TCA TCT AGC		
80	85	90
THR-GLY-TRP-ASN-GLU-THR-ILE-VAL-GLU-ASN-LEU-LEU-ALA-ASN-VAL-		
ACT GGC TGG AAT GAG ACT ATT GTT GAG AAC CTC CTG GCT AAT GTC		
95	100	105
TYR-HIS-GLN-ILE-ASN-HIS-LEU-LYS-THR-CYS-LEU-GLU-GLU-LYS-LEU-		
TAT CAT CAG ATA AAC CAT CTG AAG ACA TGC CTG GAA GAA AAA CTG		
110	115	120
GLU-LYS-GLU-ASP-PHE-THR-ARG-GLY-LYS-LEU-MET-SER-SER-LEU-HIS-		
GAG AAA GAA GAT TTC ACC AGG GGA AAA CTC ATG AGC AGT CTG CAC		
125	130	135
LEU-LYS-ARG-TYR-TYR-GLY-ARG-ILE-LEU-HIS-TYR-LEU-LYS-ALA-LYS-		
CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT TAC CTG AAG GCC AAG		
140	145	150
GLU-TYR-SER-HIS-CYS-ALA-TRP-THR-ILE-VAL-ARG-VAL-GLU-ILE-LEU-		
GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA GTG GAA ATC CTA		
155	160	165
ARG-ASN-PHE-TYR-PHE-ILE-ASN-ARG-LEU-THR-GLY-TYR-LEU-ARG-ASN-		
AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC CTC CGA AAC		

\*\*\*-  
TGA

10	20	30	40	50
MCDLPQFLQR-SSNFQSQKLL-WQLNGRLEYC-LKDRMNFDP-EEIKQLQQFQ-				
60	70	80	90	100
KEDAAITIYE-MLQNI FAIFR-QDSSSTGLNE-TIVENLLANV-YHQINHLKTC-				
110	120	130	140	150
LEEKLEKEDF-TRGKLMSSLH-LKRYYGRI LH-YLKAKEYSHC-AWTIVRVEIL-				
160				
RNFYFINRLT-GYLRN<				

## SUMMARY FLOWCHART - FORMATION OF IFNX801, X803, X804

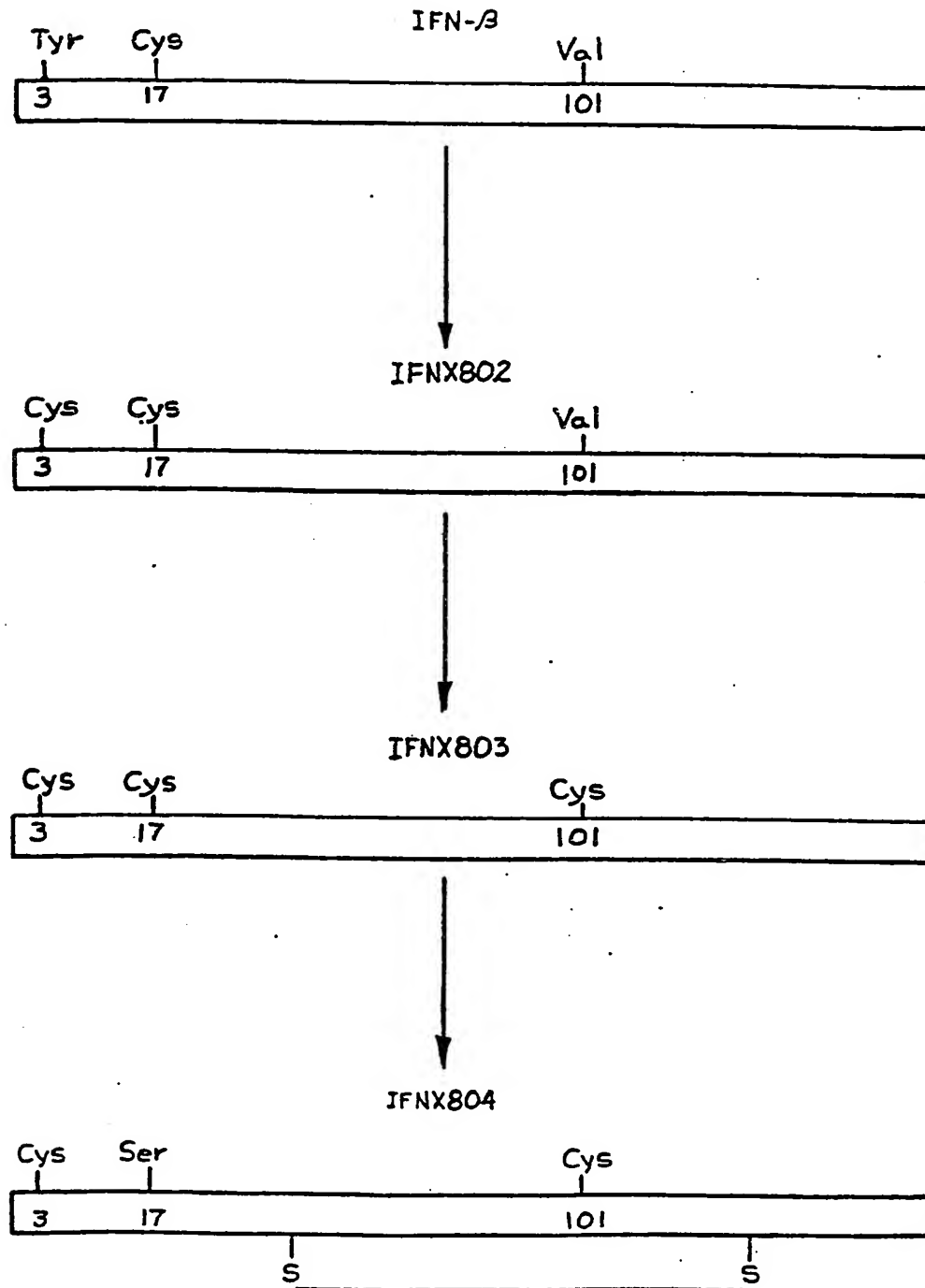


FIG. 1



RESTRICTION MAP, CONSTRUCTION I  
REPLICATIVE FORM SIZE 8.4kbp

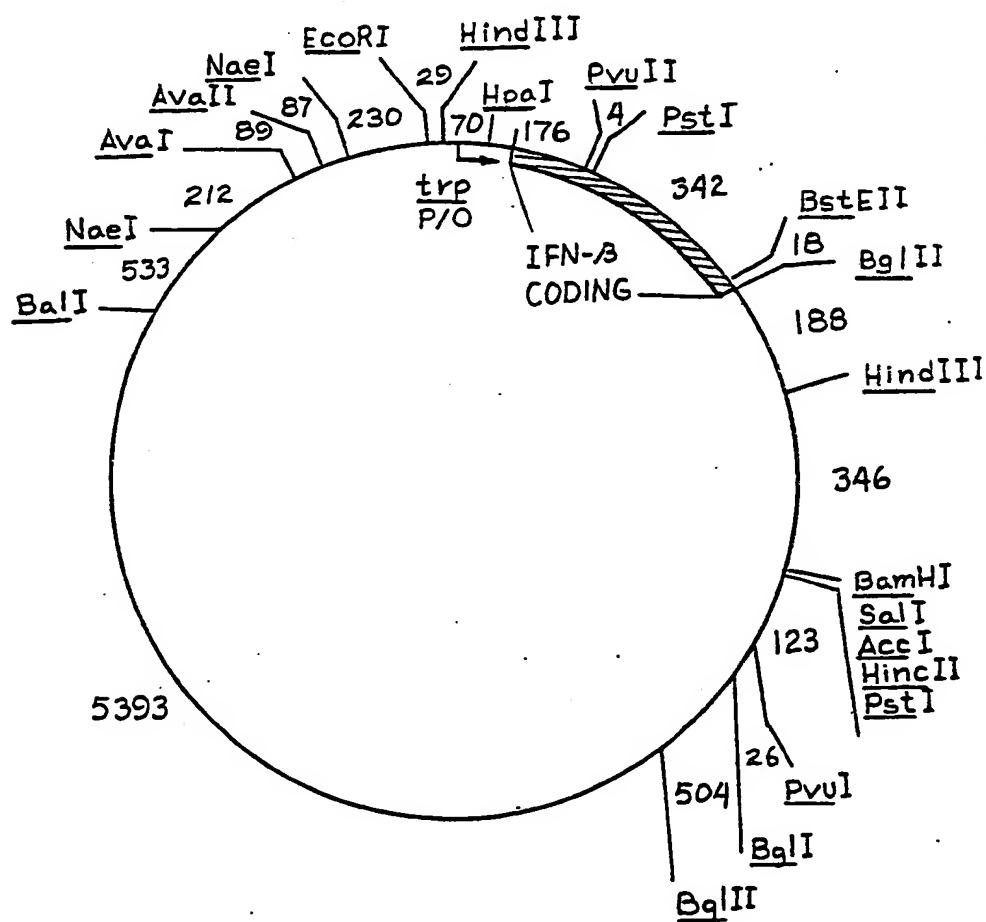
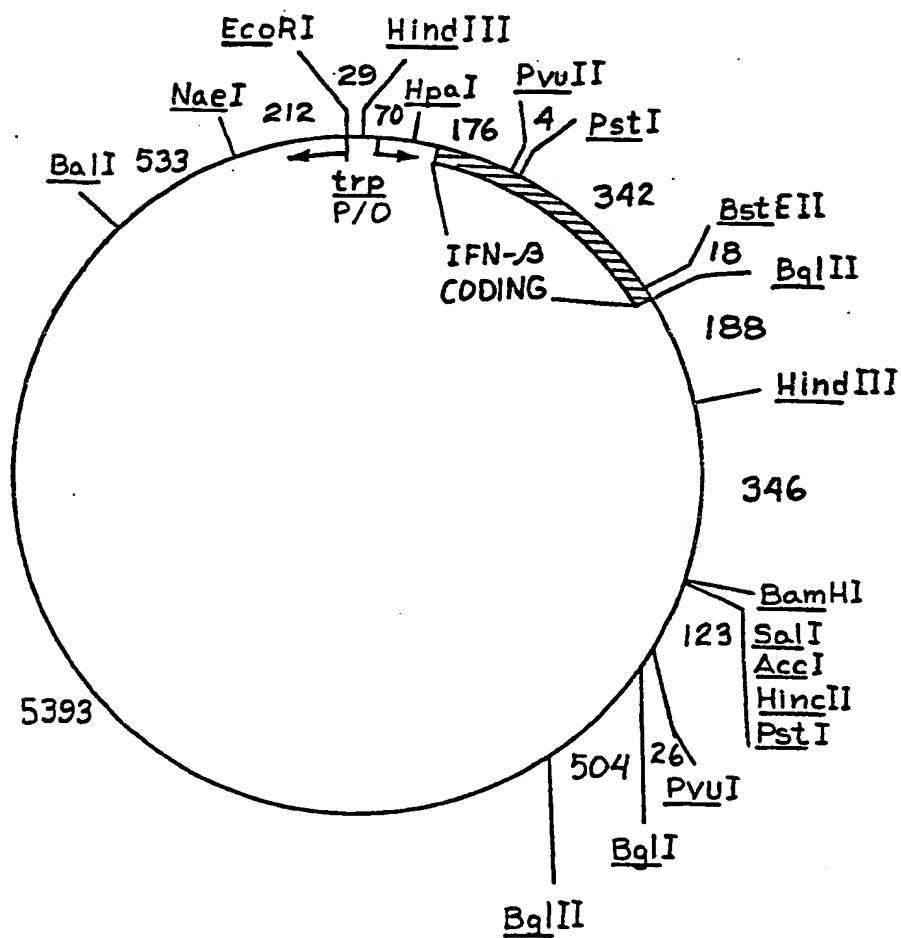


FIG. 2

RESTRICTION MAP, CONSTRUCTIONS II-V  
 REPLICATIVE FORM SIZE 8 kbp.



APPLICABLE TO M13-4AB.00  
 MJA1  
 MJA2  
 MJA3

FIG. 3

SITE DIRECTED CHANGES

1. Amino Acid 3. Human IFN- $\beta$ , Tyr  $\rightarrow$  Cys  
 Product IFNX802 (HuIFN- $\beta$ (Tyr<sup>3</sup>  $\rightarrow$  Cys<sup>3</sup>))

		1		3		5	
		Met	Ser	Tyr	Asn	Leu	
Original Sequence 5'	TCC	ATG 1	AGC	TAC —	AAC 10	TTG	3'
Primer	3'G	TAC	TCG	ACG —	TTG	A	5'
New Sequence	TCC	ATG	AGC	TGC — Cys	AAC	TTG	

FIG. 4



SITE DIRECTED CHANGES

2. Amino Acid 101. Human IFNX802, Val → Cys

Product IFNX803

(HuIFN- $\beta$ (Tyr<sup>3</sup> → Cys<sup>3</sup>) (Val<sup>101</sup> → Cys<sup>101</sup>))

	98 Leu	Lys	100 Thr	Val	102 Leu	Glu	104 Glu	
Original Sequence 5'	CTG	AAG	ACA	<u>GTC</u>	CTG	GAA	GAA	3'
Primer	AC	TTC	TGT	<u>ACG</u>	GAC	CTT	C	5'
New Sequence	CTG	AAG	ACA	<u>TGC</u> Cys	CTG	GAA	GAA	

FIG. 5



SITE DIRECTED CHANGES

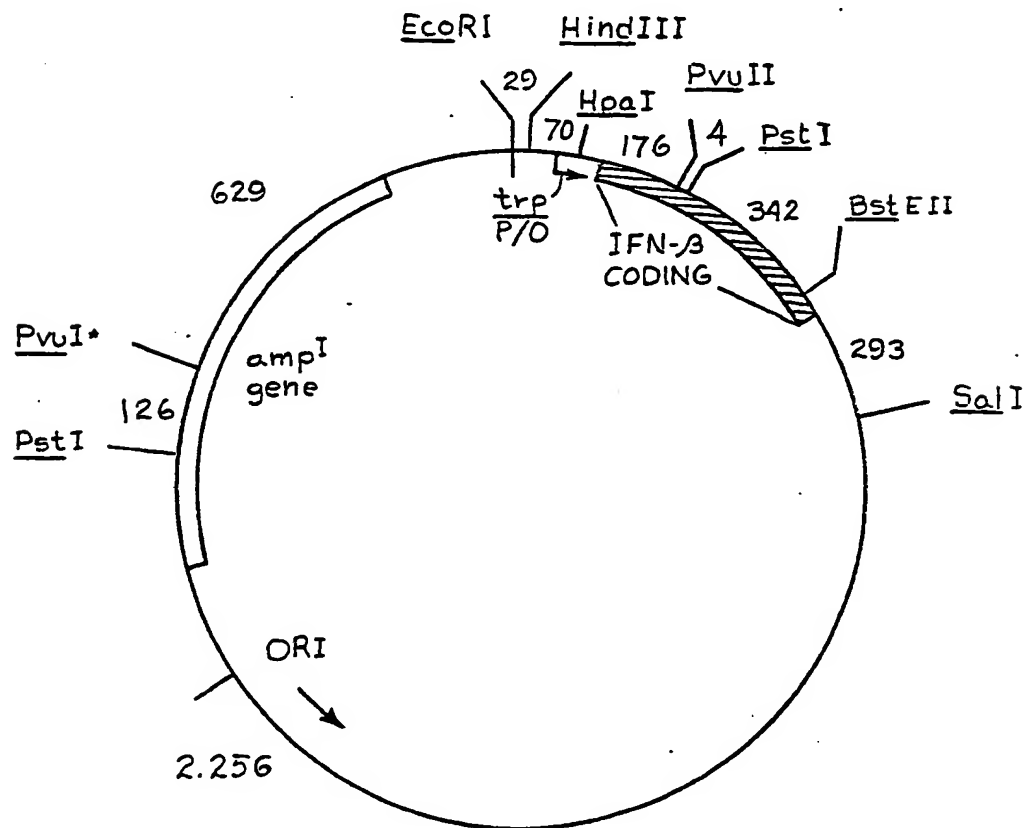
3. Amino Acid 17. Human IFN $\gamma$ 803 Cys  $\rightarrow$  Ser

Product IFN $\gamma$ 804

(HuIFN- $\beta$ (Tyr<sup>3</sup>  $\rightarrow$  Cys<sup>3</sup>) (Val<sup>101</sup>  $\rightarrow$  Cys<sup>101</sup>) (Cys<sup>17</sup>  $\rightarrow$  Ser<sup>17</sup>))

	13 Ser	Ash	15 Phe	Gln	17 Cys	Gln	
Original Sequence 5'	AGC	AAT	TTT	CAG	TGT	CAG	3'
Primer	G	TTA	AAA	GTC	TCA	GTC	5'
New Sequence	AGC	AAT	TTT	CAG	AGT Ser	CAG	

FIG. 6

RESTRICTION MAP, CONSTRUCTIONS VI, VII, VIIIPLASMID 3.92 kbp.

APPLICABLE TO PMN39-1  
PJA1  
PJA2  
PJA3

**FIG. 7**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☒ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**